

# **Cellular Engineering in Human Cells:**

## **A Synthetic Biology and Metabolic Engineering Approach**

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## Abstract

Mammalian cells are extensively used for the production of protein therapeutics due to their increased biocompatibility—when compared to those produced by bacteria, yeast, and plant eukaryotes—which stems from their ability to perform post-translational modifications.

However, one of the main drawbacks of using mammalian expression systems is that they have an absolute dependence on nine essential amino acids: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Furthermore, there are higher energy demands necessary for increased protein production. Increased citric acid cycle activity has been shown to decrease toxic byproduct accumulation from mammalian cell cultures while supplying an increased amount of ATP that is crucial to the higher energy demands necessary.

In order to address these challenges within mammalian cell culture, this work aimed at reconstructing the threonine, isoleucine, and valine biosynthetic pathways in human cell lines. A U-2 OS human osteosarcoma cell line capable of producing its own threonine was provided by the Silver Lab. Viability studies with a negative control cell line provided further evidence that this cell line was in fact expressing the threonine biosynthetic pathway. Furthermore, the genes that create the isoleucine and valine biosynthesis pathway were successfully expressed in HEK cells, a more industrially relevant cell line. Finally, the production of oxobutanoate, the first metabolite in the isoleucine and valine pathway, was proved through GC-MS analysis. In pursuance of increased citric acid cycle activity, PGC-1 $\alpha$  was overexpressed transiently in HEK cells as verified through Western blot. Finally, a mitochondrial content assay was performed in an attempt to prove more mitochondrial biogenesis as a result of PGC-1 $\alpha$  overexpression.

Advisor: Dr. Michael Betenbaugh

Reader: Dr. Marc Ostermeier

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## Chapter 1: Introduction

### 1.1 Essential Amino Acids

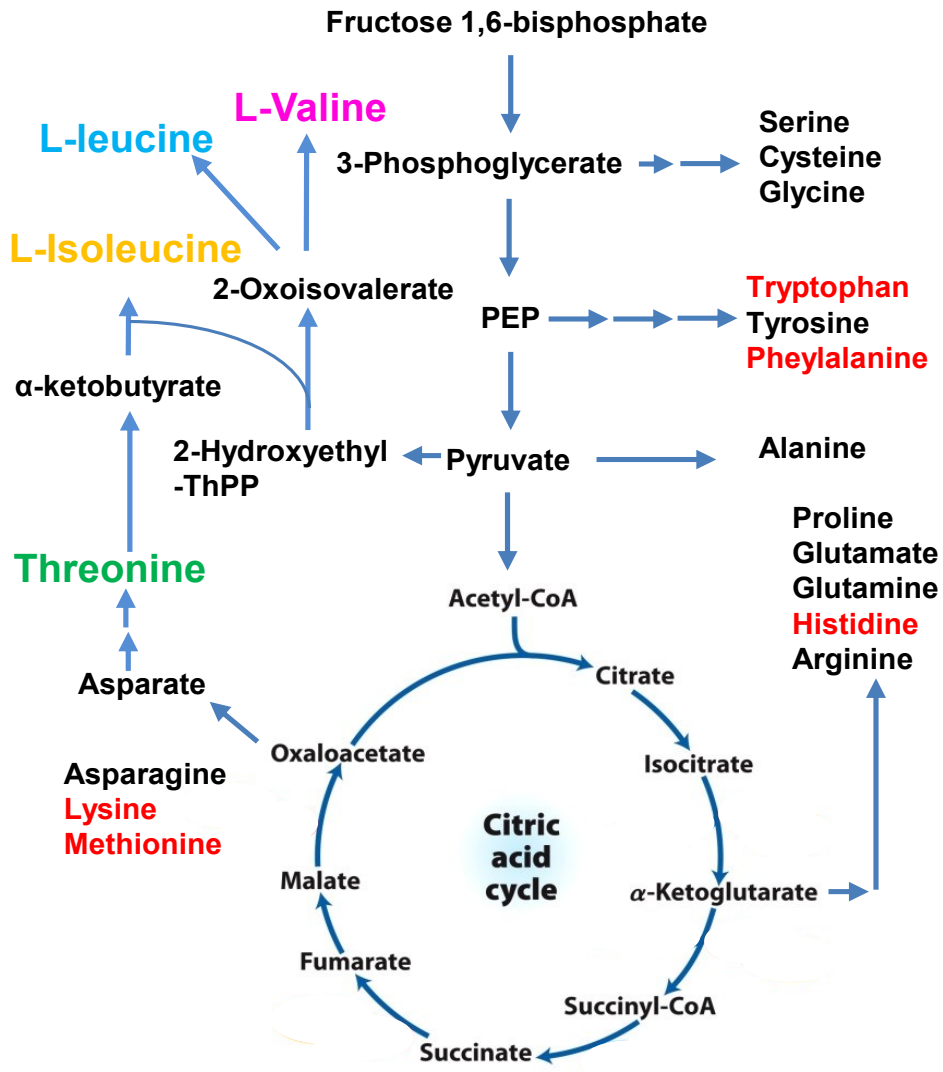
The biotechnology industry continues to rapidly expand each year due to the increasing production and use of biopharmaceuticals. In fact, biopharmaceuticals represent half of all drugs in development and 20% of all new drug approvals [1]. In 2014, the total revenue of biotechnology within the four established clusters (companies located within the United States, Europe, Australia, and Canada) was \$123B—with \$93B alone coming from the United States—a growth of over 23% from 2013 [2].

Simple organisms such as bacteria and yeast have been traditionally used for production of numerous pharmaceutical and proteins such as insulin [3]. However, it has become progressively preferred to produce recombinant proteins for biotherapeutic use in humans from mammalian cells such as Chinese Hamster Ovary (CHO) and Human Embryonic Kidney (HEK293) [4]. Mammalian cells are often favored as expression systems due to their increased biocompatibility, which stems from their ability to perform post-translational modifications. Some post translational modifications such as glycosylation and folding are essential for stability, efficacy, and avoidance of unwanted immune responses of recombinant proteins that will be used for therapeutic or research purposes [5, 6, 7]. However, these more advanced functions require compartmentalization—a key distinguishing feature between higher order eukaryotes and other organisms [8].

A significant difference between mammals (including humans) and many bacteria, yeast, and plant eukaryotes is absolute dependence on nine essential amino acids for growth and survival. These essential amino acids include: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Figure 1) [9, 10]. It is especially notable that

leucine, threonine, and valine—essential amino acids—are amongst the more abounding in the human body as evidenced by their representation of 24.4% of the amino acid content humans [11]. In addition, some amino acids require more ATP for production than others. For example, threonine and valine require an expenditure of 18.7 and 23.3 ATP respectively, while non-essential arginine expends 27.3 ATP—the same cost for leucine synthesis [12]. This supports the notion that there may be reasons other than energy costs as to why the essential amino acid biosynthetic pathways were eliminated.

It is feasible that the essential amino acid synthesis pathways can lead to the production of undesirable side-products, feedback products, or other unwelcome regulatory mechanisms. For example, the enzymes in the branched chain amino acid pathways— isoleucine, leucine, and valine—show “low substrate specificity” [13]. This warrants enzymatic multifunctionality as evidenced through synthesis of isoleucine and valine from the same enzymes. Yet, this is not necessarily beneficial in the case of isoleucine and valine because the biosynthetic enzymes involved can accept non-specific substrates in addition to the alpha-keto acids produced in the isoleucine pathways. This can lead to the production of modified amino acids such as norvaline and norleucine, in addition to the conventional amino acids. Complications can arise due to mis-aminoacylation of these alternate amino acids into the tRNA of leucine and methionine, along with their integration into proteins [14]. The engineering of select essential amino acid pathways for enhanced biopharmaceutical production in mammalian cells will be further discussed in this work.



**Figure 1:** Amino acid biosynthesis pathways. Colored: essential;  
Black: non-essential

### 1.1.1 Threonine

Threonine is an essential polar amino acid with an alcohol group and is encoded by the codons ACU, ACA, ACC, and ACG. It is produced by the emergence of aspartate from the TCA in microbes and plants—it is ingested in humans (Figure 2). Feedback inhibition occurs in this biosynthetic pathway where threonine inhibits aspartate kinase, homoserine dehydrogenase, and homoserine kinase [15]. Lysine can also inhibit aspartate kinase I which introduces a prospective bottleneck in the production of all metabolites in the aspartate family. Threonine is degraded via threonine dehydrogenase into glycine by 2-amino-3-ketobutyrate and into ammonia and 2-oxobutanoic acid by serine/threonine dehydratase [16]. Finally, the absence of a functional threonine dehydrogenase gene in humans suggests that the dehydratase maintains the predominant role [17, 18].

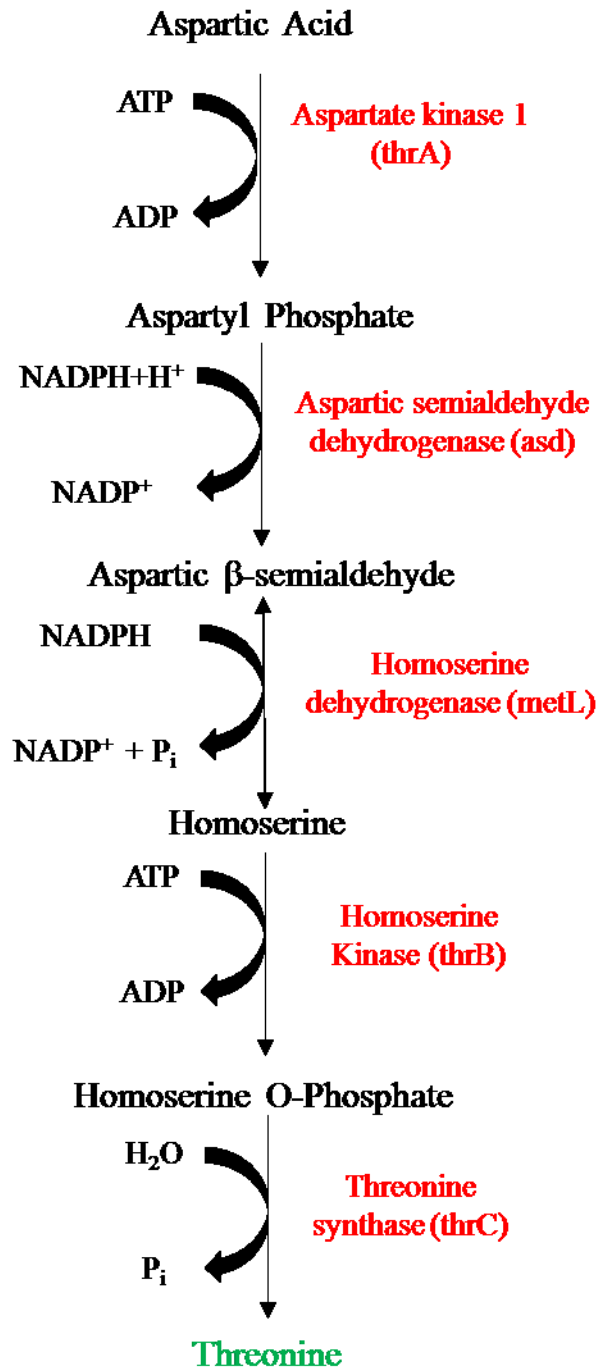


Figure 2: Threonine Biosynthetic Pathway

### 1.1.2. Valine

Valine is an essential nonpolar branched chain amino acid encoded by codons GUU, GUC, GUA and GUG. Valine is synthesized in microbes and plants through the multistep pathway shown in Figure 3 [19]. Valine is synthesized from 2-oxoisovalerate—or alpha-ketoisovalerate—by amino acid transaminase enzyme (ILVE). The same enzymes are involved in the synthesis of isoleucine. The leucine biosynthesis pathway also uses the same pathway but branches off after 2-oxovalerate. Similar to the threonine biosynthesis pathway, the first step in this pathway which is catalyzed by acetolactate synthesis undergoes feedback inhibition by both valine and leucine [20]. Valine and the other branched chain amino acids—leucine, and isoleucine—are degraded through the branched chain alpha-keto acid dehydrogenase complex. A deficiency in this complex leads to maple syrup urine disease [21]. The complex degrades the branched chain amino acids into Acyl-Co derivatives and eventually acetyl-Co or succinyl-CoA for entry in TCA cycle.

### 1.1.3. Isoleucine

Isoleucine is a hydrophobic amino acid encoded by the three codons AUU, AUC, and AUA. Threonine is converted to 2-oxobutanoate (or alpha-ketobutyrate) with an ammonia byproduct via threonine dehydratase as the initial step of isoleucine biosynthesis (Figure 3). The remaining enzymes in the pathway are collective with valine synthesis. The final step—which involves the conversion of 3-methyl-2-oxopentanoate (or 2-oxo-3-methylvalerate/keto-methylvalerate)—is catalyzed by amino acid transaminase enzyme (ILVE). Threonine dehydratase, the first enzyme in the pathway, is also subject to feedback inhibition by isoleucine and feedback activation by valine [22].

There is an alternative isoleucine pathway that can completely bypass the threonine pathway and the utilization of threonine dehydratase—along with avoiding the challenge of feedback inhibition. Isoleucine biosynthesis in *G. sulfurreducens* [23] and cyanobacterial species [24] uses acetyl-CoA and pyruvate to produce 2-oxobutanoate (alpha-ketobutyrate) through citramalate. In this pathway, citramalate is generated from acetyl-CoA and pyruvate by the CimA enzyme. (Figure 4). This is then followed by conversion to citraconate, then beta-methyl-D-malate, and finally 2-oxobutanoate—the first dedicated substrate in the isoleucine pathway. This pathway can proceed using the leucine pathway enzymes and serves to generate NADH in addition to 2-oxobutanoate [25]. Thus, the 2-oxobutanoate precursor can be synthesized from either citramalate or threonine.

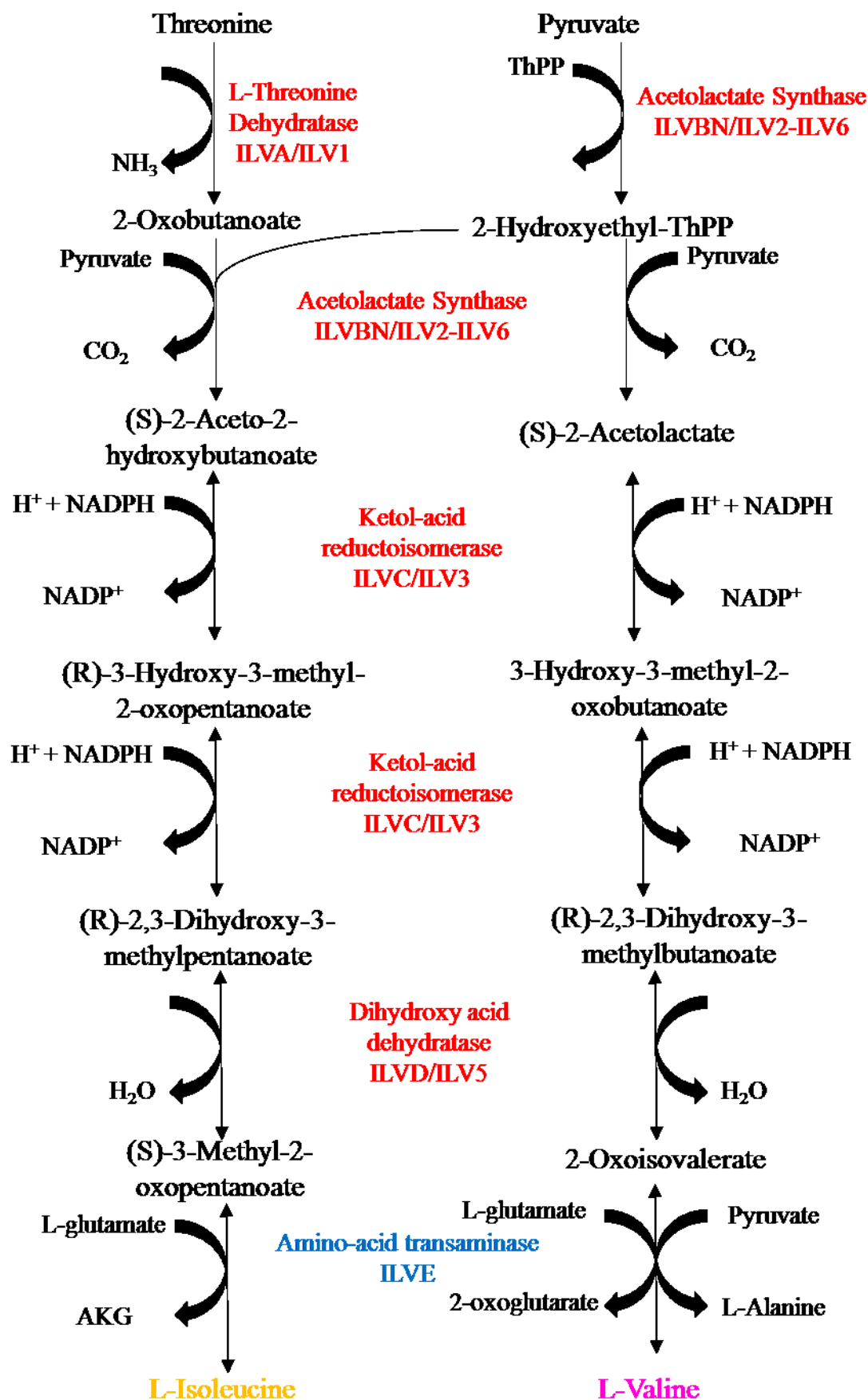
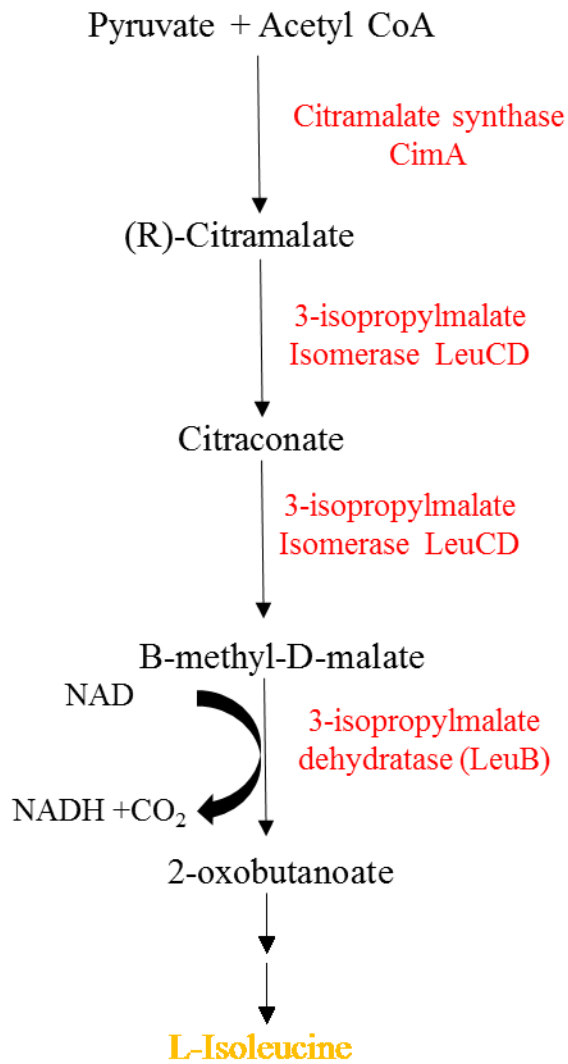


Figure 3: Isoleucine and Valine Biosynthetic Pathway

Blue: Present in Mammalian Red: Must be engineered

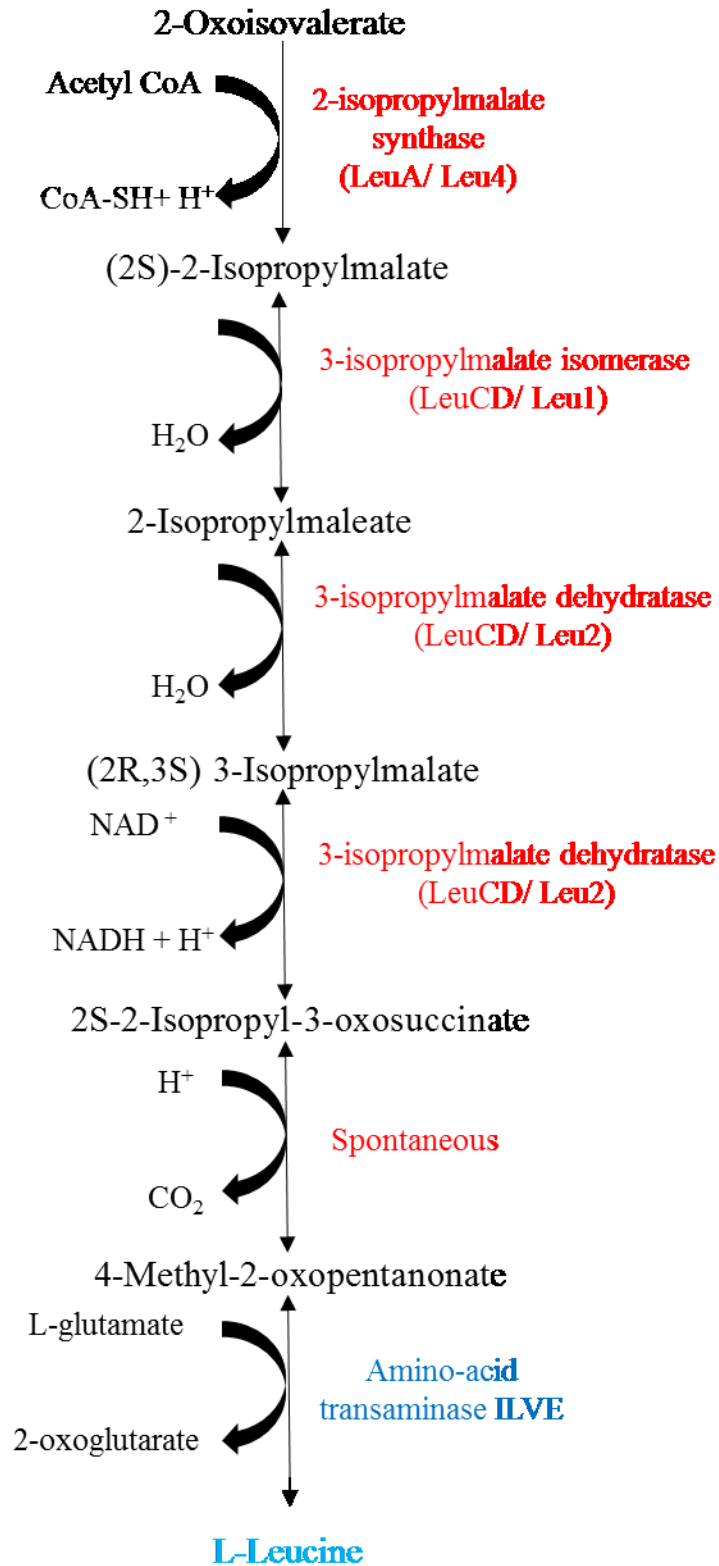




**Figure 4:** Citramalate Pathway

#### 1.1.4 Leucine

Leucine is another essential branched chain amino acid with an isobutyl side chain resulting in its hydrophobicity. Is it coded for by the following six codons: UUA, UUG, UUC, CUC, CUA, and CUG. Leucine is synthesized from pyruvic acid in multiple steps—with the first ones being shared with the valine synthesis pathway. At the metabolic intermediate 2-oxovalerate (alpha-ketoisovalerate), the leucine pathway diverges with the production of 2-isopropylmalate by isopropylmalate synthase otherwise known as LeuA (Figure 5). After a series of steps, leucine is synthesized by the amino acid transferase (ILVE) that is common to the isoleucine and valine pathways as well. Leucine regulates the pathway through feedback inhibition of the first step catalyzed by LeuA. Additionally, leucine regulates the first step in the shared valine pathway catalyzed by acetolactate synthase [26].



**Figure 5:** Leucine Biosynthetic Pathway

## 1.2 PGC-1 $\alpha$

Peroxisome proliferator-activated receptor (PPAR)- $\gamma$  coactivator (PGC)-1 $\alpha$  is a transcriptional coactivator that regulates genes involved in energy metabolism through stimulation of mitochondrial biogenesis and function along with other key metabolic functions [27]. PGC-1 $\alpha$  acts as a coactivator for a number of transcription factors involved in mitochondrial biogenesis such as nuclear respiratory factor (NRF) 1 and 2, along with estrogen-related receptor (ERR)  $\alpha$ ,  $\beta$ , and  $\gamma$  [28, 29, 30]. Additionally, PGC-1 $\alpha$  is a coactivator for a number of other transcription factors that are involved in fatty acid oxidation, gluconeogenesis, and triglyceride metabolism [31, 32, 33, 34, 35, 36]. PGC-1 $\alpha$  was first identified as a transcription coactivator of adaptive thermogenesis induced by a cold environment. Adaptive thermogenesis is the process of heat dissipation in response to environmental changes in temperature and diet [37, 38].

PGC-1 $\alpha$  also plays a role in skeletal muscle fiber conversion. PGC-1 $\alpha$  expression is induced by short and long term exercise in humans and rodents. This results in an increase in the ratio of glycolytic fibers to oxidative fibers [39, 40, 41, 42]. PGC-1 $\alpha$  similarly participates in heart development. An increase in expression level is followed by a considerable surge in mitochondria biogenesis and oxidative metabolism in a developing heart [43]. In addition, PGC-1 $\alpha$  overexpression can initiate the expression of gluconeogenic genes in primary hepatocytes [44]. This has been further validated through studies in PGC-1 $\alpha$  knockout mice which showed fasting hypoglycemia during conditions of compromised gluconeogenic gene expression [45, 35]. Furthermore, PGC-1 $\alpha$  plays a role in Type 2 diabetes through downregulation in muscle tissues [29, 46, 47]. In addition, a polymorphism of the PGC-1 $\alpha$  gene that results in reduced activity has been associated with an increased risk for type 2 diabetes [48, 49]. It is therefore

believed that reduced PGC-1 $\alpha$  activity can lead to insulin resistance. Finally, the use of PGC-1 $\alpha$  overexpression for upregulation of mitochondrial oxidative metabolism will be furthered discussed in subsequent sections.

## Chapter 2: Engineering of Essential Amino Acid Pathways into U-2 OS Cells

### 2.1 Introduction

As previously mentioned, mammalian cells lack the capabilities to synthesize essential amino acids. If these amino acid requirements could be removed from mammalian cell culture, then the production of biotherapeutics could lower the costs associated with their culture media. The simplification of mammalian cell culture formulations could lower the costs of mammalian biomanufacturing and in thus lead to reduced biopharmaceutical costs.

#### 2.1.1 Threonine Biosynthetic Pathway

The threonine biosynthetic pathway consists of five steps beginning with aspartic acid. The first reaction involves the conversion of aspartic acid into aspartyl phosphate through the transfer of a phosphate group from ATP by aspartate kinase 1. The *Escherichia coli* gene for aspartate kinase 1 is thrA. The second step uses aspartic semialdehyde dehydrogenase—its respective *E. coli* gene is known as asd—to convert the aspartyl phosphate into aspartic  $\beta$ -semialdehyde. It utilizes an NADPH and a hydrogen ion and releases NADP<sup>+</sup> and a phosphate group as a byproduct. The third step consists of the conversion of aspartic  $\beta$ -semialdehyde into homoserine through homoserine dehydrogenase (metL). It utilizes an NDAPH and a hydrogen ion and releases an NADP<sup>+</sup> as a byproduct. The fourth step converts homoserine into homoserine o-phosphate through the transfer of a phosphate group from ATP by homoserine kinase (thrB). The final step uses threonine synthase (thrC) to convert homoserine o-phosphate into threonine—it utilizes a water molecule and gives off a phosphate group as a byproduct [50].

### 2.1.2 Isoleucine and Leucine Biosynthetic Pathway

The isoleucine and leucine biosynthetic pathways share the same enzymes. The first step in the production of isoleucine begins with the conversion of threonine to 2-oxobutanoate (or alpha-ketobutyrate) through threonine dehydratase (ILVA/ILV1) with ammonia as the byproduct. The subsequent step converts 2-oxobutanoate with pyruvate to (S)-2-aceto-2-hydroxybutanoate with CO<sub>2</sub> as a byproduct via acetolactate synthase (ILVBN/ILV2-6). The third step is the conversion to (R)-3-hydroxy-3-methyl-2-oxopentanoate with NADPH and a hydrogen ion via ketol-acid reductoisomerase (ILVC/ILV3) with NADP<sup>+</sup> as a byproduct. The fourth step also uses ketol-acid reductoisomerase (ILVC/ILV3) to create (R)-2,3-Dihydroxy-3-methylpentanoate. The fifth step converts (R)-2,3-Dihydroxy-3-methylpentanoate into (S)-3-Methyl-2-oxopentanoate via dihydroxy acid dehydratase (ILVD/ILV5) with water as a byproduct. The final step towards isoleucine biosynthesis is the conversion of (S)-3-Methyl-2-oxopentanoate utilizing glutamate via amino-acid transaminase (ILVE) with alpha ketoglutarate as the byproduct [50].

The first step in valine biosynthesis is the conversion of pyruvate in 2-hydroxyethyl-ThPP with ThPP as a cofactor via acetolactate synthase (ILVBN/ILV2-ILV6). The second step also involves the use of acetolactate synthase (ILVBN/ILV2-ILV6) with pyruvate as a cofactor to convert 2-hydroxyethyl-ThPP into (S)-2-acetolactate with CO<sub>2</sub> as a byproduct. The following step converts (s)-2-acetolactate into 3-hydroxy-3-methyl-2-oxobutanoate with a hydrogen ion and NADPH via ketol-acid reductoisomerase (ILVC/ILV3) producing NADP<sup>+</sup> as a byproduct. The fourth step also utilizes ketol-acid reductoisomerase (ILVC/ILV3) to create (R)-2,3-dihydroxy-3-methylbutanoate. The fifth step converts that into oxoisovalerate with water as a byproduct through dihydroxy dehydratase (ILVD/ILV5). The final step uses glutamate and

pyruvate via amino-acid transaminase (ILVE) with 2-oxoglutarate and alanine as byproducts to produce valine [50].

## 2.2 Materials and Methods

### 2.2.1 Cell Culture and Cell Lines

Aseptic cell culture was performed in a biosafety cabinet (Labconco, Kansas City, MO). Cells were incubated at 37° C and 5% CO<sub>2</sub> in humidified incubators (Thermo Fisher Scientific, Carlsbad, CA). U-2 OS and U-2 OS threonine prototroph adherent cell lines were kindly provided by Wade Hicks from Dr. Pamela Silver's lab at Harvard University. U-2 OS cells are a human osteosarcoma cell line. To create the threonine prototroph, the U-2 OS cells were transformed in a one-step transfection with the plasmid shown in Figure 6 and selected through threonine-deprived Dulbecco's modified Eagle's medium (DMEM) media (Thermo Fisher Scientific, Carlsbad, CA). Cells were supplemented with 10% dialyzed Fetal Bovine Serum (Thermo Fisher Scientific, Carlsbad, CA) and 2.5 mM aspartic acid (Sigma Aldrich). Untransfected U-2 OS cells were used as a control cell line for various experiments.

### 2.2.2 Viability Studies

U-2 OS cells and U-2 OS threonine prototrophic cells were plated at 450,000 cells/well in six well tissue culture treated plates (Corning, Corning, NY). They were each placed under three conditions: 4.5 g/L Dulbecco's modified Eagle's medium (DMEM) media supplemented with 10% Fetal Bovine Serum, threonine-deprived Dulbecco's modified Eagle's medium (DMEM) media supplemented with dialyzed Fetal Bovine Serum, and threonine-deprived DMEM media supplemented with dialyzed Fetal Bovine Serum and 2.5 mM aspartic acid (Sigma Aldrich). Cell counts were taken every 24 hours for six days. One well from each condition was harvested

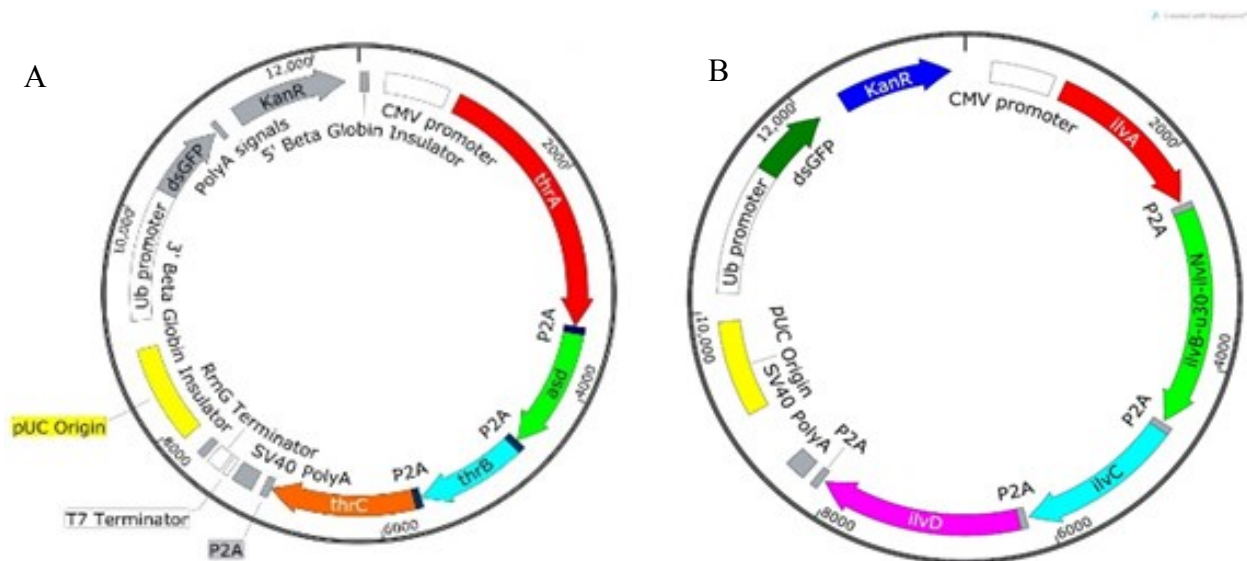


using the media from each well to detach the cells from the well of interest. Triplicate live/dead cell counts from each of well were performed every day using trypan blue solution (Corning, Corning, NY). Cells were counted on a light microscope (Zeiss, Oberkochen, Germany) using a hemocytometer (Electron Microscopy Sciences, Hatfield, PA).

## 2.2.3 DNA Constructs and Transfection

### 2.2.3.1 Bacterial Genes

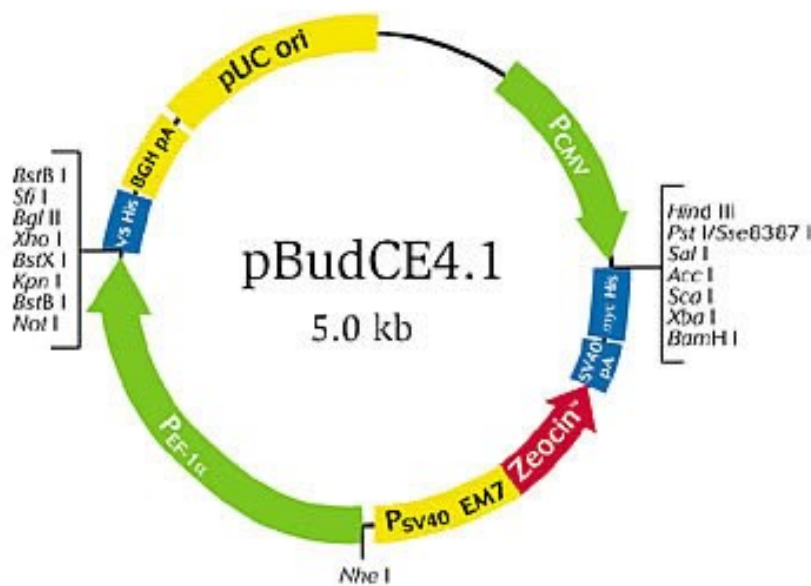
The genes needed to complete the threonine pathway—*thrA*, *thrB*, *thrC*, and *asd*—and the isoleucine-valine pathway—*ilvA*, *ilvB*, *ilvC*, *ilvD*, and *ilvN*—were amplified from *E. coli* by performing polymerase chain reaction (PCR) and inserted into the pFUSE-like vector using Gibson assembly. All genes were separated by the porcine teschovirus 2A peptide sequence inserted between genes to allow the use of a single CMV promoter and a polycistronic assembly. These two vectors were kindly provided to us by the Silver lab (Figure 6).



**Figure 6:** Polycistronic transcripts containing genes for threonine, isoleucine, and valine. (A) Vector containing genes for bacterial threonine biosynthetic pathway. (B) Vector containing genes for bacterial isoleucine and valine biosynthetic pathway.

### 2.2.3.2 Yeast Genes

The genes needed to complete the isoleucine valine-pathway—ILV1, ILV2, ILV3, ILV5, and ILV6—were amplified from *S. cerevisiae* by performing polymerase chain reaction (PCR) and individually inserted into the pBudCE4.1 plasmid shown in Figure 7 (Thermo Fisher Scientific, Carlsbad, CA). The individual genes are all under the EF-1 $\alpha$  promoter using the restriction sites NotI and XhoI for a total of five plasmids.



**Figure 7:** pBudCE4.1 plasmid from Thermo Fisher Scientific

### 2.2.3.3 Transfections

Lipofectamine 3000 reagent (Thermo Fisher Scientific, Carlsbad, CA) was used to transfect the vectors into both U-2 OS and U-2OS threonine prototrophic cell lines in Opti-MEM I media (Thermo Fisher Scientific, Carlsbad, CA) according to the manufacturers protocol.

#### 2.2.4 $^{13}\text{C}$ Aspartic Acid

Universally labeled  $^{13}\text{C}$  aspartic acid was purchased from Sigma-Aldrich. A 30mM stock solution of the aspartic acid tracers were prepared by dissolving 100 mg of the  $^{13}\text{C}$  aspartic acid into 25 mL of water and heating to 37°C for four hours. The solution was then filter sterilized with a 0.2  $\mu\text{m}$  filter. A concentration of 1 mM aspartic acid was used in the culture medium for labeling experiments.

#### 2.2.5 GC-MS Analysis

Intracellular metabolites were extracted from six well tissue culture treated plates and ten centimeter culture treated plates (Corning, Corning, NY) by washing twice with 4°C cold saline at 9g/L NaCl. Then cell metabolism was quenched with cold methanol and the plates were incubated on ice for five minutes. Afterwards, the cells were scraped off of the plate surfaces. Next the suspension was transferred into glass tubes with teflon-sealed caps. The next step involved the addition of 1.5 mL of chloroform and vigorously vortexing for ten seconds. Subsequently, 1.5 mL of water was added to suspend cell debris which was then followed by vigorous vortexing for one minute. Afterwards, the samples were stored overnight at 4°C. The next day, samples were centrifuged at 2000 rpm and 4°C for 20 minutes. The upper phase with methanol and water was then transferred to two 1.5 mL Eppendorf tubes and evaporated to dryness under nitrogen gas at 37°C.

Derivatization and GC-MS analysis was graciously performed at the Antoniewicz lab. After drying, the metabolites were suspended in 50  $\mu\text{L}$  of 2% wt methoxylamine hydrochloride in pyridine and incubated at 37°C for 90 minutes on a heating block. After 70  $\mu\text{L}$  of MTBSTFA + 1% TBDMS was added to the samples, they were incubated at 60°C for 30 minutes. The next

step involved incubating the samples at room temperature overnight. Then, the derivatized samples were centrifuged for two minutes at 14,000g. Then supernatants were then transferred to GC vials for the GC-MS analysis. Sample volumes of 1  $\mu$ L were injected in split mode with split ratio ranging from 1:2 to 1:5. Over the 75-minute run time, the GC oven temperature was varied as follows: temperature was held at 70°C for two minutes, then increased to 140°C at a rate of 3°C per minute, then increased to 150°C at 1°C per minute, then increased to 242°C at 3°C per minute, and finally increased to 280°C at 10° per minute. This temperature was maintained constant until the end of the 75-minute run time. The mass spectra of selected metabolite fragments were collected in SIR mode.

The GC-MS analysis was performed using an Agilent 7890A GC equipped with a DB-5ms capillary column (30 m x 0.25 mm i.d. x 0.25  $\mu$ m; Agilent J&W Scientific) for analysis of aqueous metabolites. The GC instrumented interfaces with a Waters Quatro Micro GC-MS/MS (Milford, MA) operating under electron impact ionization at 70 eV and 200°C ion source temperature. The injection port and interface temperatures were 250°C. Helium flow was maintained at 1mL/min. Mass spectra were recorded in selected ion recording (SIR) mode with 30 ms dwell time [51].

## 2.3 Results

### 2.3.1 Viability Studies

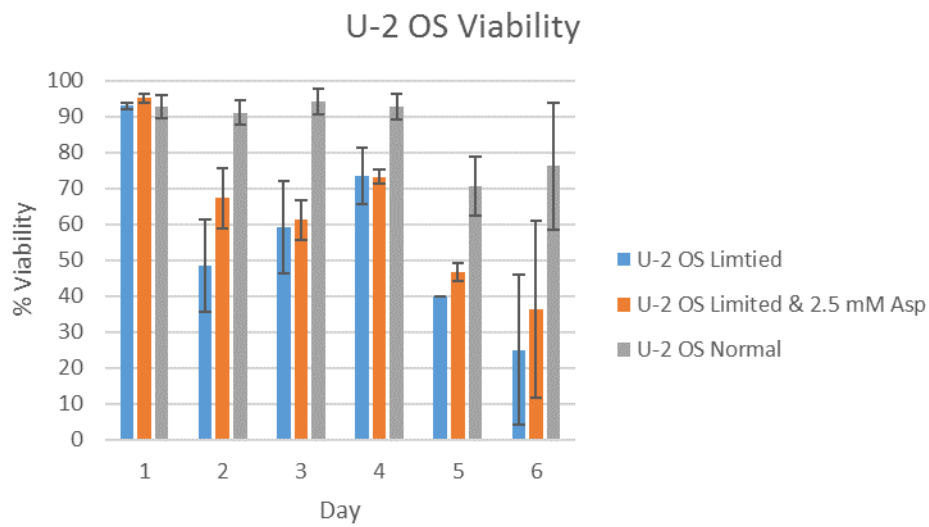
To determine the impact of essential amino acid depletion on U-2 OS cells, cells were cultured under three conditions: threonine limited media, threonine limited media supplemented with 2.5 mM aspartic acid, and normal DMEM media. The same culture practices were also

performed on a U-2 OS threonine prototrophic cell line in order to determine the viability effects as well as to verify the presence of the threonine biosynthetic pathway.

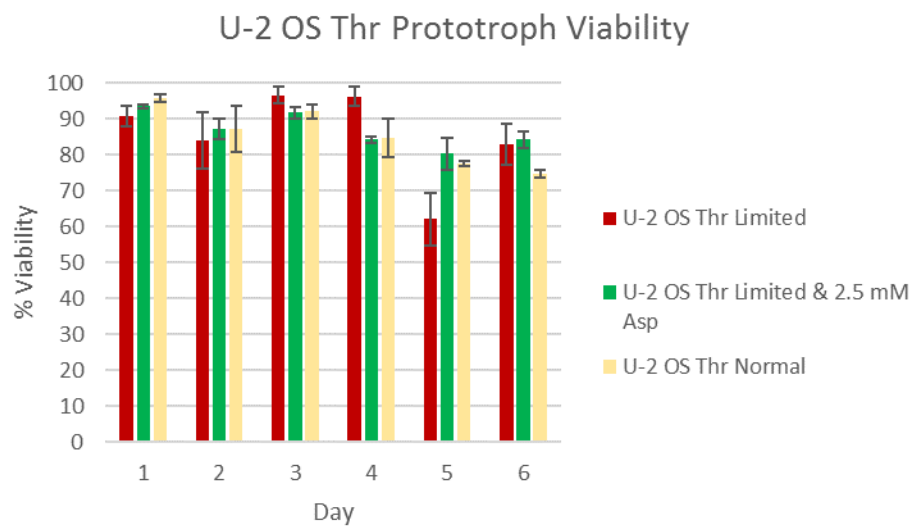
The U-2 OS cells had similar viabilities on the first day (Figure 8); however, on Day 2, there was a significant decrease in the viability of the cells cultured without threonine—including those supplemented with aspartic acid—when compared to the cells cultured on normal DMEM media. While the viability improves slightly for the U-2 OS cells on days three and four, the viability is significantly less than the cells on normal media. Furthermore, on days five and six, the viability of the cells cultured on threonine limited media and threonine limited media with aspartic acid falls drastically. While the viability for the U-2 OS cells cultured on normal media also fall, the viability is much higher than those cells on limited media. In summary, U-2 OS viability is not sustainable on threonine depleted media.

The U-2 OS threonine prototrophic cells maintained similar viabilities across all three conditions (Figure 9). On Day 1, the viabilities of threonine limited media, threonine limited media supplemented with aspartic acid, and normal media are nearly indistinguishable. By day four, the viabilities begin to fall slightly. While the threonine prototrophic cells have a lower viability than the other conditions at Day 5, it is evident that the cells that have been supplemented with aspartic acid maintain higher viability by the last days of the study.

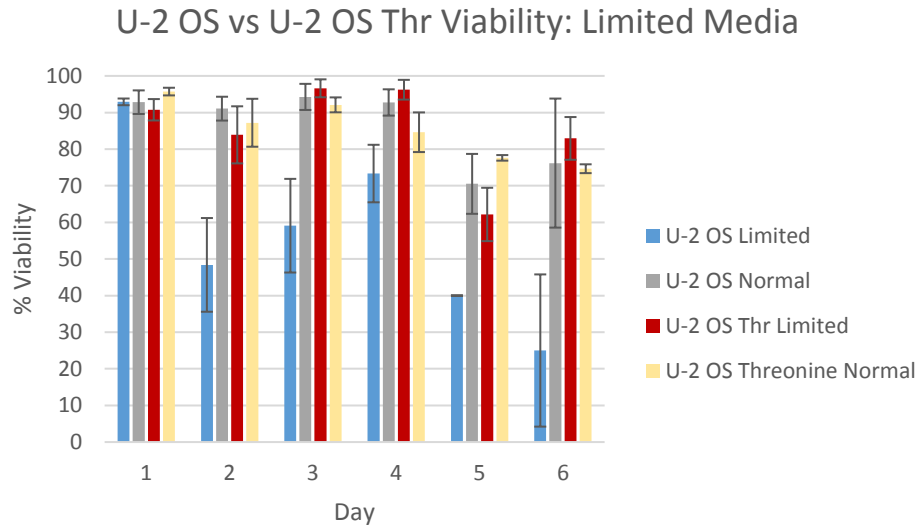
The most telling results are when the U-2 OS and U-2 OS threonine prototrophic cells are compared (Figure 10-11). On Day 1, the U-2 OS cells under all conditions are very similar to the threonine prototroph cell line. However, by Day 2, the viability of the U-2 OS cells under threonine limited media are much lower than those under the other conditions. U-2 OS cells in normal media maintain a high viability—with a slight decrease that begins on Day 5—along with the U-2 OS threonine prototrophic cells in threonine limited media and normal media.



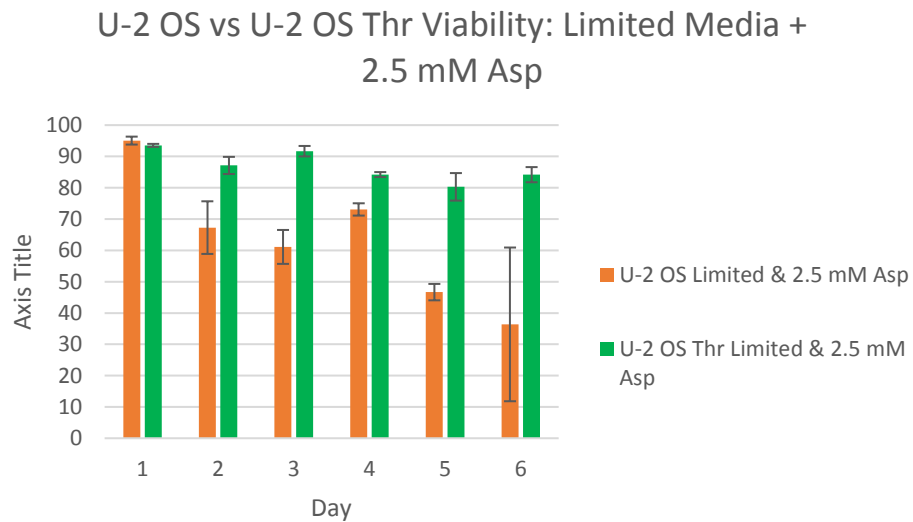
**Figure 8:** U-2 OS viability under threonine limited media, threonine limited media supplemented with 2.5 mM aspartic acid, and normal media.



**Figure 9:** U-2 OS threonine prototroph viability under threonine limited media, threonine limited media supplemented with 2.5 mM aspartic acid, and normal media.

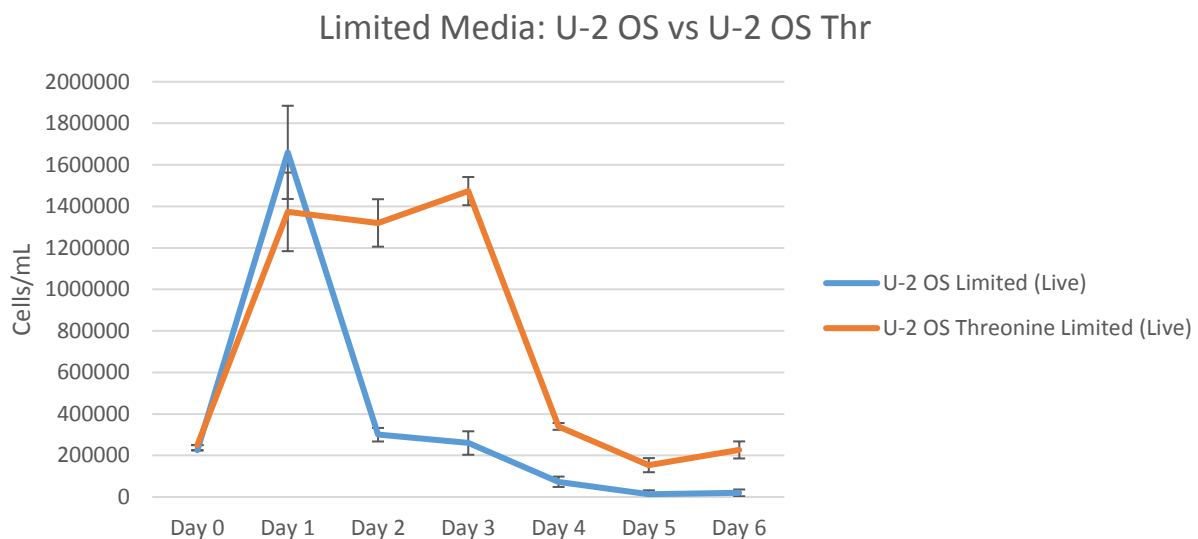


**Figure 10:** U-2 OS vs U-2 OS threonine prototroph viability under threonine limited media, and normal media.



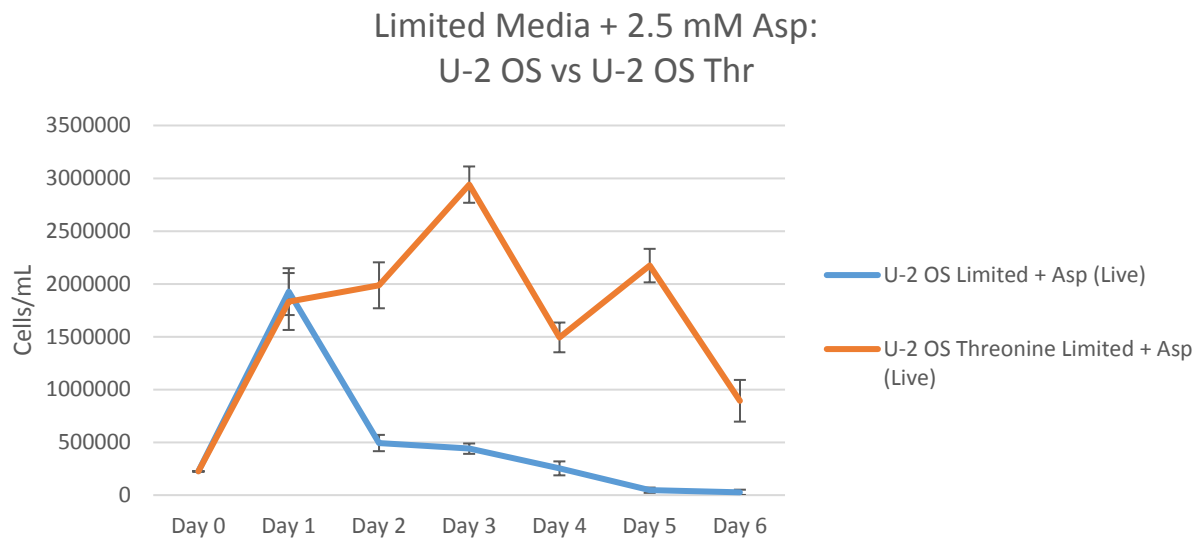
**Figure 11:** U-2 OS vs U-2 OS threonine prototroph viability under threonine limited media supplemented with 2.5 mM aspartic acid.

When looking at the cell counts, the raw numbers support the viability analysis. When comparing the cell densities of live cells under the tested conditions, it is evident that the U-2 OS cells cannot survive without threonine while the prototrophic cell line does. Figure 12 shows similar growth between both cells on the first day, but by the second day the U-2 OS cells greatly decrease in number while the prototrophic cells remain steady. While by day four the U-2 OS Thr cell line decreased in density, it was maintained higher than the U-2 OS cell line for the duration of the study. When supplementing the limited media with Aspartate, there was a more notable difference between the two cell lines. The threonine prototrophic cells maintained a much higher cell density throughout the experiment (Figure 13). Finally, when comparing the two cell lines under normal media conditions, they show a similar growth pattern, with the difference being the day in which the bulk of the growth occurs (Figure 14).

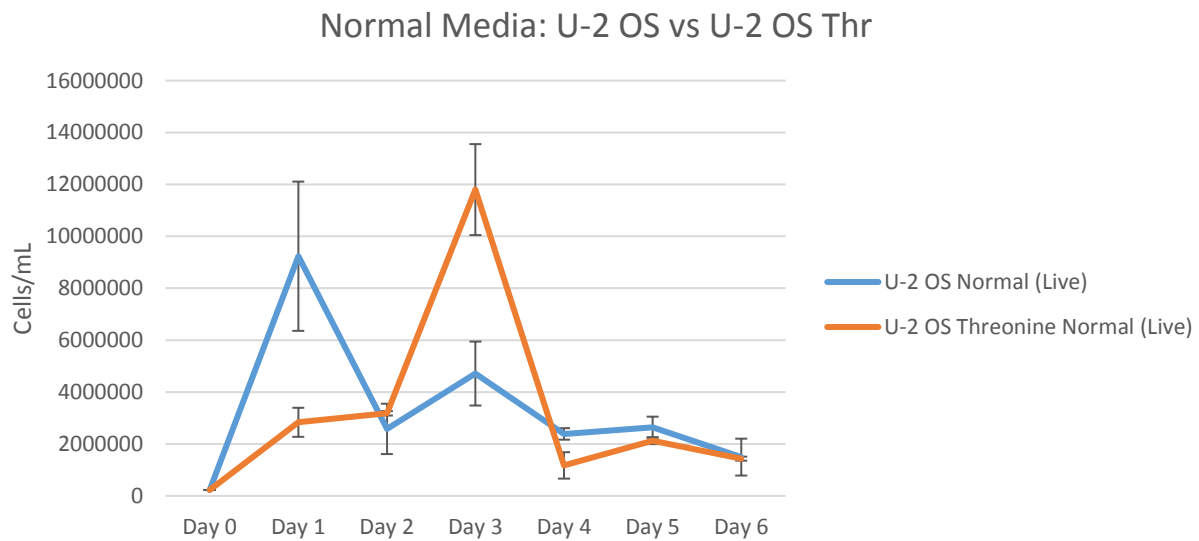


**Figure 12:** U-2 OS vs U-2 OS threonine prototroph cell density under threonine limited media.





**Figure 13:** U-2 OS vs U-2 OS threonine prototroph cell density under threonine limited media supplemented with 2.5 mM aspartic acid.



**Figure 14:** U-2 OS vs U-2 OS threonine prototroph cell density under normal media.

### 2.3.2 GC-MS and $^{13}\text{C}$ Aspartic Acid Analysis

The results of these studies are pending. They will be presented in the future work as the data is still being analyzed.

## 2.4 Discussion

The viability studies demonstrated that U-2 OS cells—a human cell line—cannot maintain desirable viability when they are cultured without threonine. This confirms that mammalian cells cannot maintain viable without essential amino acids—even if they are lacking only threonine as in this case. Perhaps the most important deduction that can be drawn from this viability study is that the U-2 OS cell line that was transfected with the threonine biosynthetic cell line and selected for using threonine limited media may in fact be producing its own threonine. The self-production of threonine is also supported by the fact that supplementation with aspartic acid maintains the highest viability by the end of the study. Aspartic acid feeds into the threonine biosynthetic pathway that was transfected and therefore requires aspartic acid to function. This study also supports the selection of method that was used to generate the threonine prototrophic cell line. In the continuation of engineering a human cell line capable of producing all of the essential amino acids, selection through essential amino acid derived media may be a more effective approach than the traditional method involving antibiotic resistance.

## Chapter 3: Engineering of Essential Amino Acid Pathways in HEK293t Cells

### 3.1 Introduction

Monoclonal antibodies, peptides, and recombinant proteins are some of the most widely produced biomolecules manufactured by the pharmaceutical industry [52, 53]. These therapeutics can be produced using bacterial, yeast, plant, insect, and mammalian expression systems [54]. Expression systems are chosen depending on the proteins of interest. In the case of producing biopharmaceuticals, mammalian cell lines are preferred due to their ability to produce large, complex proteins such as membrane proteins and perform post translational modifications—most importantly glycosylation—that are compatible with humans [52, 54, 55]. The most common non-human mammalian cell line used for biotherapeutic production are Chinese hamster ovary (CHO) cells; however, this cell line can produce post translational modifications that are not expressed in humans [56]. This presents challenges because non-human cell lines must be screened during production in order to identify clones with compatible glycan profiles [57].

The challenges with non-human, mammalian expression systems have given rise to the increases interest in human cell lines such as human embryonic kidney (HEK293) for biotherapeutic production due to their ability to synthesize proteins that are more analogous to those in humans [57]. Human cells have an advanced protein glycosylation ability as evidenced by approximately 2% of the human transcriptome encoding for proteins involved in glycosylation [58]. When producing proteins to treat human disease, using proteins that originate from human cells can help decrease the probability of inducing undesired immune responses as compared to those originating from non-human sources. While HEK293 cells include the

oligosaccharide synthetic capabilities, they lack the minimalist cell capabilities of creating fundamental amino acid building blocks from basic nutrients that are present in bacteria, plants, and fungi—that is, the essential amino acids: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine [59].

Due to human cells lacking amino acid minimalist capabilities, the biopharmaceutical industry has optimized cell culture media formulations to provide all of the necessary nutrients such as the essential amino acids, glucose, growth factors, and buffers. However, adding these nutrients is extremely costly. With the arguments previously stated, it is evident that it would be beneficial to re-create the biosynthetic pathways for the essential amino acids into a human cell line—in this case the widely used HEK293 cell line—in order to maximize their efficiency and productivity while reducing culture costs in an industrial setting. While there are still a number of disadvantages to using human cell lines such as the potential susceptibility to human viral contamination, and the fact that clinical experience is still not as widespread, there are a number of benefits to using HEK293 cells. These cells can be easily grown in suspension, they divide quickly, they are responsive to a number of transfection methods, and are very efficient at protein production [60, 61].

HEK293 cells have also recently been used to produce therapeutics approved by the Federal Drug Administration (FDA) and/or the European Medicines Agency (EMA). Some of these drugs include drotrecogin alfa for severe septic shock treatment (XIGRIS®; Eli Lilly Corporation, Indianapolis, IN), recombinant factor IX Fc fusion protein (rFIXIC; Biogen, Cambridge, MA) and human cell line recombinant factor VIII (human-cl rhFVIII; NUWIQ®; Octapharma, Lachen, Switzerland) used to treat hemophilia A, recombinant factor VIII Fc fusion protein (rFVIII-Fc; Biogen, Cambridge, MA) used to treat hemophilia B, and dulaglutide

(TRULICITY®; Eli Lilly, Indianapolis, IN) used to treat type 2 diabetes [62]. Engineering the biosynthetic pathways for the essential amino acids into HEK cells would increase efficiency and lower the costs of HEK293 cell therapeutic production for existing treatments and future proteins of interest.

## 3.2 Materials and Methods

### 3.2.1 Cell Culture and Cell Lines

Aseptic cell culture was performed in a biosafety cabinet (Labconco, Kansas City, MO). Cells were incubated at 37° C and 5% CO<sub>2</sub> in humidified incubators (Thermo Fisher Scientific, Carlsbad, CA). HEK293 cells were used for these experiments. They were maintained in 4.5 g/L glucose Dulbecco's modified Eagle's medium (DMEM) media (Thermo Fisher Scientific, Carlsbad, CA).

### 3.2.2 Viability Studies

HEK cells were plated at 450,000 cells/well in six well tissue culture treated plates (Corning, Corning, NY). They were placed under three conditions: 4.5 g/L Dulbecco's modified Eagle's medium (DMEM) media supplemented with 10% Fetal Bovine Serum; threonine, isoleucine, leucine, and valine deprived Dulbecco's modified Eagle's medium (DMEM) media supplemented with dialyzed Fetal Bovine Serum; and threonine, isoleucine, leucine, and valine deprived Dulbecco's modified Eagle's medium (DMEM) media supplemented with dialyzed Fetal Bovine Serum supplemented with 2.5 mM aspartic acid (Sigma Aldrich). Cell counts were taken every 24 hours for six days. One well from each condition was harvested using the media from each well to detach the cells from the well of interest. Triplicate live/dead cell counts from each of well were performed every day using trypan blue solution (Corning, Corning, NY). Cells

were counted on a light microscope (Zeiss, Oberkochen, Germany) using a hemocytometer (Electron Microscopy Sciences, Hatfield, PA).

### 3.2.3 DNA Constructs and Transfection

#### 3.2.3.1 Bacterial Genes

The genes needed to complete the threonine pathway—*thrA*, *thrB*, *thrC*, and *asd*—and the isoleucine-valine pathway—ILVA, ILVB, ILVC, ILVD, and ILVN—were amplified from *E. coli* by performing polymerase chain reaction (PCR) and inserted into the pFUSE-like vector using Gibson assembly. All genes were separated by the porcine teschovirus 2A peptide sequence inserted between genes to allow for the use of a single CMV promoter and a polycistronic assembly. These two vectors were kindly provided to us by the Silver lab (Figure 6).

#### 3.2.3.2 Yeast Genes

The genes needed to complete the isoleucine valine-pathway—ILV1, ILV2, ILV3, ILV5, and ILV6—were amplified from *S. cerevisiae* by performing polymerase chain reaction (PCR) and individually inserted into the pBudCE4.1 plasmid shown in Figure 7 (Thermo Fisher Scientific, Carlsbad, CA) previously.

#### 3.2.3.3 Transfection

Lipofectamine 3000 reagent (Thermo Fisher Scientific, Carlsbad, CA) was used to transfect the vectors into HEK293 cells in Opti-MEM I media (Thermo Fisher Scientific, Carlsbad, CA) according to the manufacturers protocol.

### 3.2.4 Western Blot

Cells were lysed using 1x radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology; Danvers, MA) containing Complete Mini ethylenediaminetetraacetic acid-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The precipitate was separated by centrifugation, and the soluble protein lysate solution was collected. Protein concentrations were determined using a BCA protein assay kit (Pierce; Rockford, IL). Samples were incubated with 6X SDS loading dye for five minutes at 95°C. Equal amounts of protein were loaded into wells of 10% acrylamide gels and separated by electrophoresis for approximately two hours at 100 volts. Proteins were transferred to a nitrocellulose membrane (Bio-Rad; Hercules, CA) for 75 minutes at 100 volts. Membranes were blocked for two hours in TBST solution containing 5% milk. A mouse anti-His tag primary antibody, and an anti-mouse HRP-conjugated secondary antibody (Cell Signaling Technology) were used. In addition, a mouse anti-V5 tag primary antibody, and an anti-mouse HRP-conjugated secondary antibody (Cell Signaling Technology) were also used. Membranes were visualized using a ChemiDoc UV transilluminator (Bio-Rad, Hercules, CA) and SuperSignal<sup>TM</sup> West Pico Chemiluminescent Substrate (Thermo Fisher, Carlsbad, CA).

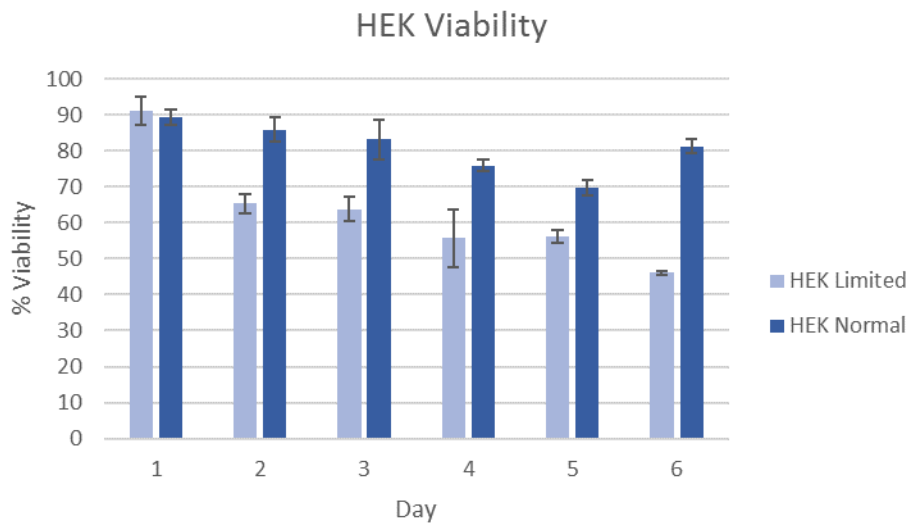
### 3.2.5 GC-MS Analysis

Intracellular metabolite extraction and GC-MS analysis was performed as previously outlined in section 2.2.5.

### 3.3 Results

#### 3.3.1 Viability

To determine the impact of essential amino acid depletion on HEK cells, cells were cultured on threonine, isoleucine, valine, and leucine depleted media, and normal DMEM media (Figure 12). By Day 2 of the study, the HEK cells cultured on essential amino acid limited media had a much lower viability than those on normal media that is supplemented with the essential amino acids. This trend continues throughout the duration of the six days with the viability of the cells on limited media dropping down to below 50% while those on normal DMEM are approximately 80%.

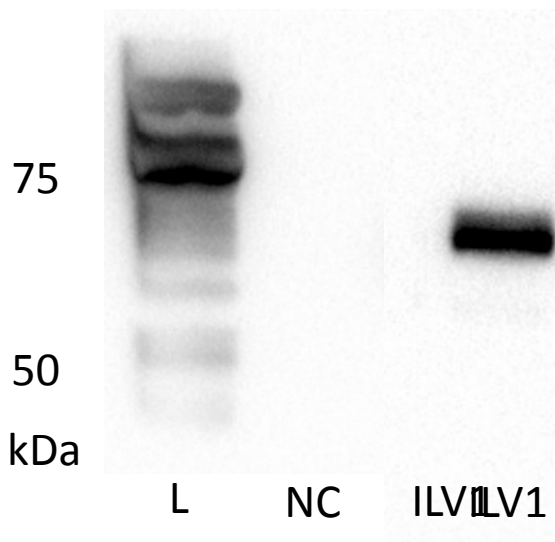


**Figure 15:** HEK viability under threonine, isoleucine, valine, and leucine limited media, and normal media.

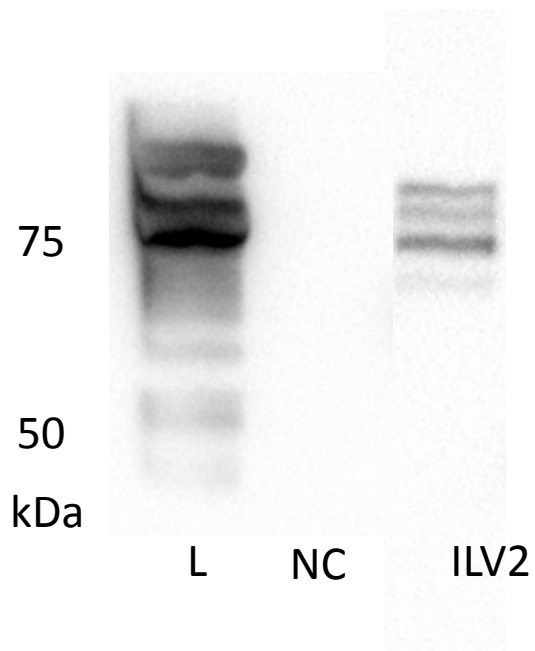


### 3.3.2 Western Blots

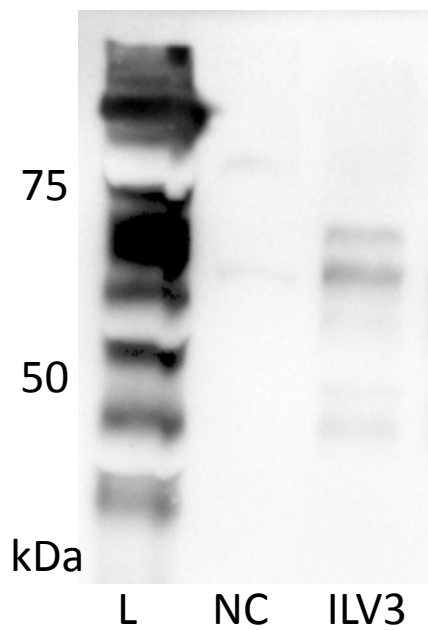
SDS-PAGE was performed on protein lysate from untransfected HEK cells, as well as HEK cells transfected with ILV1, ILV2, ILV3, ILV5, and ILV6 individually. Both an anti-His and an anti-V5 antibody was used, and the results indicate that each of these genes. ILV1, ILV2, ILV3, ILV5, ILV6 expression can be seen in Figures 13, 14, 15, 16, and 16 respectively.



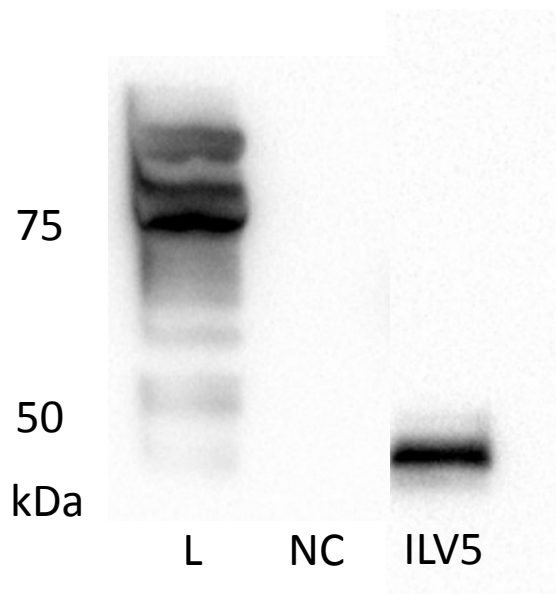
**Figure 16:** Western Blot of ILV1 with anti-V5 antibody. L: ladder; NC: untransfected negative control; ILV1  $\approx$  64 kDa



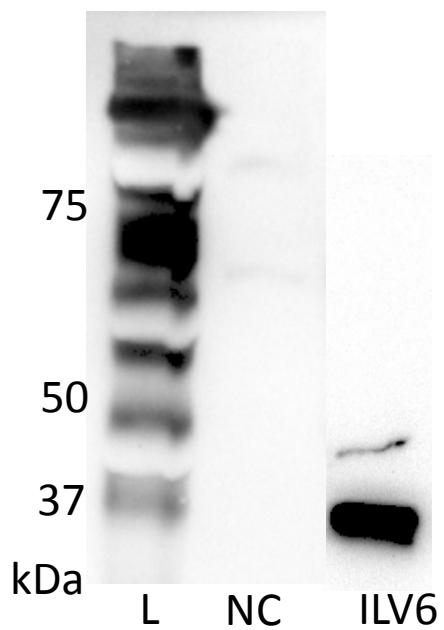
**Figure 17:** Western Blot of ILV2 with anti-V5 antibody. L: ladder; NC: untransfected negative control; ILV2  $\approx$  75 kDa



**Figure 18:** Western Blot of ILV3 with anti-His antibody. L: ladder; NC: untransfected negative control; ILV3  $\approx$  63 kDa



**Figure 19:** Western Blot of ILV5 with anti-V5 antibody. L: ladder; NC: untransfected negative control; ILV5  $\approx$  44 kDa

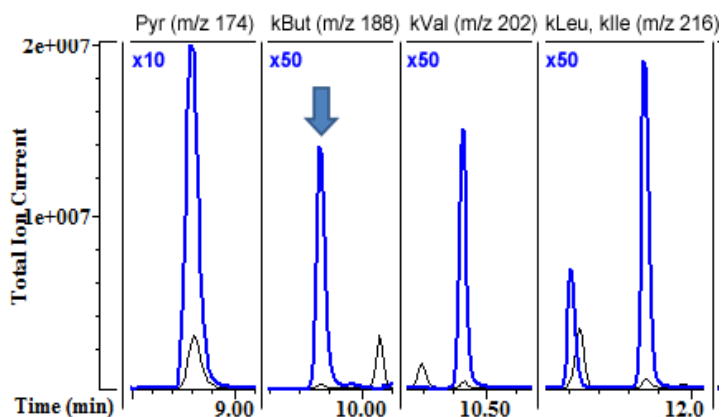


**Figure 20:** Western Blot of ILV6 with anti-His antibody. L: ladder; NC: untransfected negative control; ILV 6  $\approx$  34 kDa

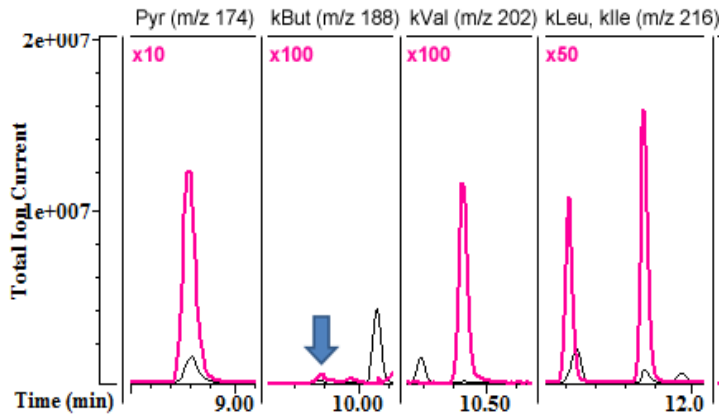
### 3.3.3 GC-MS Analysis

Pure samples of 2-oxobutanoate, (S)-3-methyl-2-oxopentanoate, and 2-oxoisovalerate (Sigma Aldrich) were individually analyzed through the GC-MS in order to characterize their respective profiles for comparison to both HEK cells transfected with ILV1 and untransfected HEK cells. Figure 18 indicates the presence of 2-oxobutanoate—or alpha-ketobutyrate—in the transfected cells with no peak in the untransfected cells. In addition, it has been thought that the final step of valine and isoleucine biosynthesis was present in mammalian cells, but it was verified through this analysis as peaks for (S)-3-methyl-2-oxopentanoate (kIle), 2-Oxoisovalerate (kVal), and 4-methyl-2-oxopentanoate (kLeu) were detected.

#### (A) ILV1 overexpressing cells



#### (B) Negative control



**Figure 21:** GC-MS analysis of intracellular metabolites in HEK. (A) analysis of cells transfected with ILV1 and (B) untransfected negative control HEK cells. The arrow shows the presence of 2-oxobutanoate or alpha-ketobutyrate in cells transfected with ILV1.

### 3.4 Discussion

The viability study demonstrated the reliance of human cells on essential amino acids. There was approximately a 30% difference in viability between cells lacking four essential amino acids and the cells which were adequately nourished with normal DMEM media. Although viability of the amino acid deficient cells drops below 50%, by Day 6, HEK cells can be cultured for three days and maintain approximately 65% viability. This could be useful for future work involving recreating the entire isoleucine, leucine, and valine biosynthetic pathways if an amino acid reduction selection method were to be used.

The western blots for ILV1, ILV2, ILV3, ILV5, and ILV6 demonstrate that HEK cells are capable of expressing their respective genes and producing the enzymes that catalyze the steps in the isoleucine and valine biosynthetic pathways. It was made evident that ILV1 was expressed in Figure 13 through the band that appears between the 50 kDa and 75 kDa ladder bands at approximately 64 kDa—the size of threonine dehydratase. ILV2 expression was proven in Figure 14 through the band that appears at nearly 75 kDa which corresponds to the approximate size of 75 kDa consistent with acetolactate synthase size. It was also clear that ILV3 was expressed in Figure 15 through the strong band in between the 50 kDa and 75 kDa ladder which is consistent with the size of ketol-acid reductoisomerase which is approximately 63 kDa. ILV5 expression (Figure 16) was also proven through the presence of a band right below 50 kDa which is consistent with the approximate 44 kDa size of dihydroxy acid dehydratase. Finally, ILV6 (Figure 17) expression was proven with the presence of a band right below 37 kDa consistent with the size of the regulatory subunit of acetolactate synthase—approximately 34 kDa.

The GC-MS analysis demonstrated that ILV1, threonine dehydratase, took threonine from the culture media and catalyzed the conversion of threonine to 2-oxobutanoate. It also confirmed that the precursors to isoleucine, valine, and leucine are synthesized within mammalian cells through amino-acid transaminase as previously assumed.

## Chapter 4: Overexpression of PGC-1 $\alpha$

### 4.1 Introduction

As previously mentioned, mammalian cells have become the preferred expression system to produce biotherapeutics. There are a number of approaches that can be employed in order to increase protein production. The overall goal is to use a metabolic engineering approach by overexpressing PGC-1 $\alpha$  in order to increase cellular capability through up-regulating mitochondrial oxidative metabolism. The benefits of enhancing productivity in a mammalian cell line would involve decreased lead times and costs of drug manufacturing. Previous work has demonstrated that high-producing Chinese hamster ovary (CHO) cell lines exhibit enhanced citric acid cycle activity when compared to its low producing counterparts [43].

Increased citric acid cycle activity has been shown to decrease toxic byproduct accumulation while also supplying an increased amount of ATP that is crucial to the higher energy demands necessary for increased protein production [43]. It is hypothesized that a global regulator of mitochondrial metabolism such as PGC-1 $\alpha$  may help drive oxidative metabolism [27]. PGC-1 $\alpha$  overexpression has been shown to increase mitochondrial content, enhance cellular respiration, and suppress reactive oxygen species accumulation in a number of different tissues [43, 63, 64]. It is beneficial to study the effects of PGC-1 $\alpha$  in a prominent industrial cell line such as HEK293. The ultimate goal is to identify the metabolic impacts of PGC-1 $\alpha$  overexpression and to increase citric acid cycle activity.

## 4.2 Materials and Methods

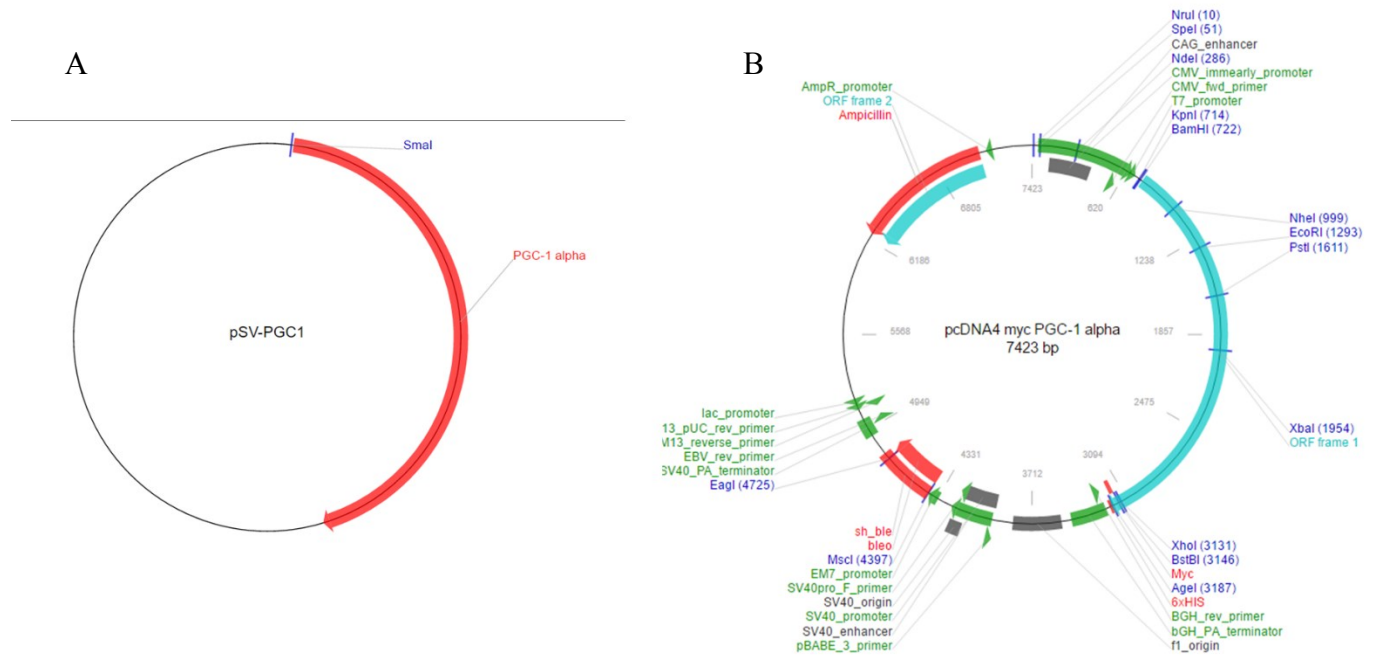
### 4.2.1 Cell Culture and Cell Line

Aseptic cell culture was performed in a biosafety cabinet (Labconco, Kansas City, MO). Cells were incubated at 37° C and 5% CO<sub>2</sub> in humidified incubators (Thermo Fisher Scientific, Carlsbad, CA). HEK293 cells were used for these experiments. They were maintained in 4.5 g/L glucose Dulbecco's modified Eagle's medium (DMEM) media (Thermo Fisher Scientific, Carlsbad, CA).

### 4.2.2 DNA Constructs and Transfection

Plasmids containing PGC-1 $\alpha$  were acquired from Addgene. Two plasmids were obtained, an untagged PGC-1 $\alpha$  plasmid (Figure 19 A)—pSV-PGC1 (Addgene plasmid #3)—and a myc tagged PGC1- $\alpha$  plasmid (Figure 19 B)—pcDNA4 myc PGC1- $\alpha$  (Addgene plasmid # 10974). Lipofectamine 3000 reagent (Thermo Fisher Scientific, Carlsbad, CA) was used to transfect the vectors into HEK293 cells in Opti-MEM I media (Thermo Fisher Scientific, Carlsbad, CA) according to the manufacturers protocol. Stable cell lines were selected for 14 days using 150  $\mu$ g/mL Zeocin (Thermo Fisher Scientific).





**Figure 22:** PGC1- $\alpha$  plasmids (A) untagged PGC1- $\alpha$  plasmid (B) Myc tagged PGC1- $\alpha$  plasmid

### 4.2.3 Western Blot

Cells were lysed using 1x radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology; Danvers, MA) containing Complete Mini ethylenediaminetetraacetic acid-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The precipitate was separated by centrifugation, and the soluble protein lysate solution was collected. Protein concentrations were determined using a BCA protein assay kit (Pierce; Rockford, IL). Samples were incubated with 6X SDS loading dye for five minutes at 95°C. Equal amounts of protein were loaded into wells of 10% acrylamide gels and separated by electrophoresis for approximately two hours at 100 volts. Proteins were transferred to a nitrocellulose membrane (Bio-Rad; Hercules, CA) for 75 minutes at 100 volts. Membranes were blocked for two hours in TBST solution containing 5% milk. A mouse anti-myc tag primary antibody, and an anti-mouse HRP-conjugated secondary antibody (Cell Signaling Technology) were used. In addition, a

rabbit anti-PCG-1 $\alpha$  tag primary antibody, and an anti-rabbit HRP-conjugated secondary antibody (Cell Signaling Technology) were also used. Membranes were visualized using a ChemiDoc UV transilluminator (Bio-Rad, Hercules, CA) and SuperSignal<sup>TM</sup> West Pico Chemiluminescent Substrate (Thermo Fisher, Carlsbad, CA).

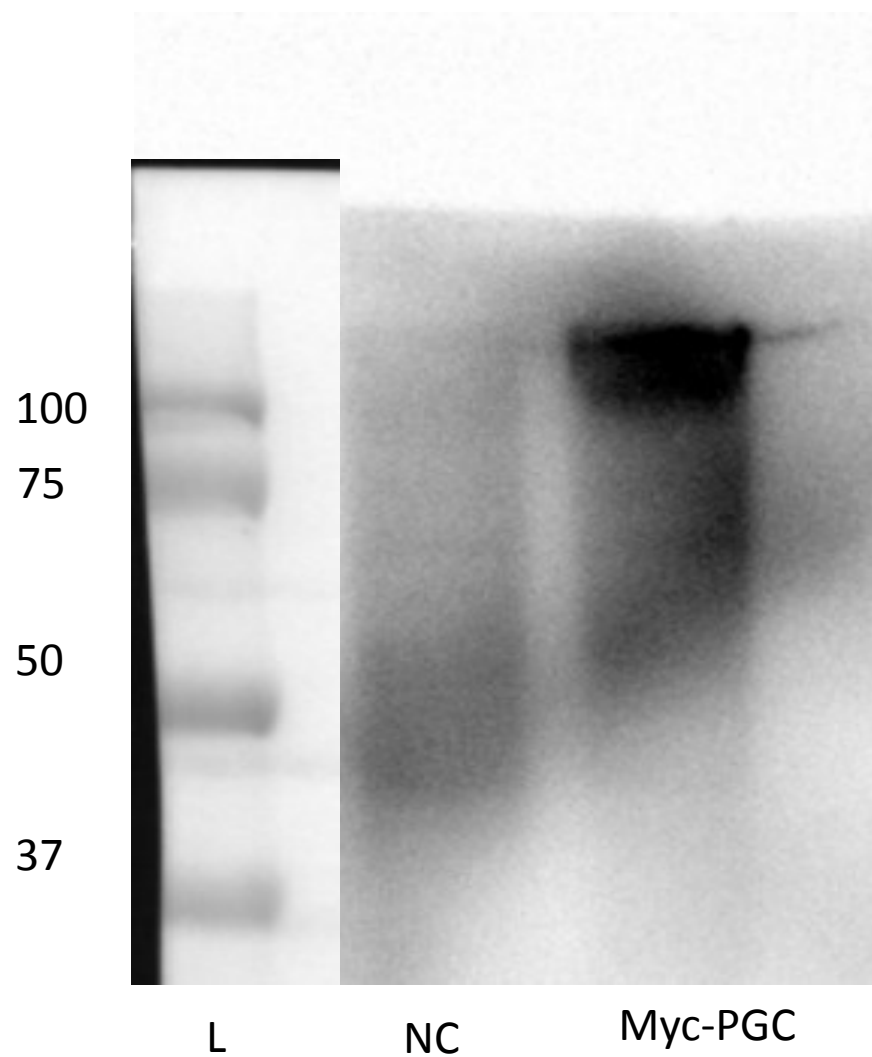
#### 4.2.4 Mitotracker

MitoTracker<sup>®</sup> Red FM (Thermo Fisher, Carlsbad, CA) was used to stain mitochondria and assess the effect of PCG1- $\alpha$  overexpression on mitochondria biogenesis. Stable pool transfected cells were plated onto 96 well tissue culture treated plates (Corning, Corning, NY) and stained according to the manufacturers protocol. Fluorescence was measured at 644 nm using the GloMax<sup>®</sup> multimode plate reader (Promega). Cell counts were also taken in order to normalize fluorescence values. One well from each condition was harvested using the media from each well to detach the cells from the well of interest. Duplicate live/dead cell counts from each of well were performed using trypan blue solution (Corning, Corning, NY). They were counted on a light microscope (Zeiss, Oberkochen, Germany) using a hemocytometer (Electron Microscopy Sciences, Hatfield, PA).

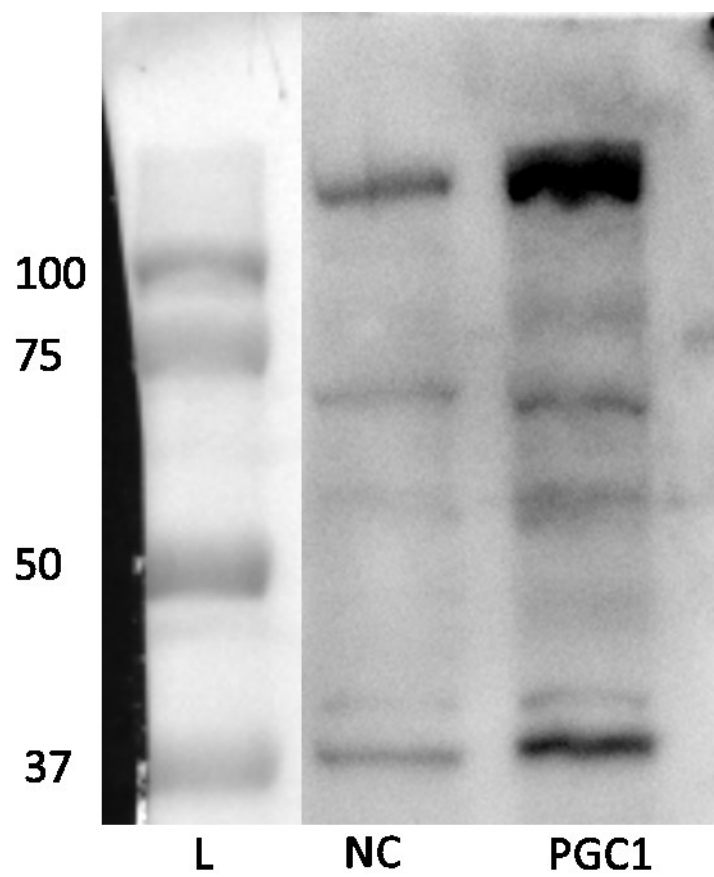
### 4.3 Results

#### 4.3.1 Western Blots

SDS-PAGE was performed on protein lysate from untransfected HEK cells, as well as HEK cells transfected with an untagged PGC-1 $\alpha$  plasmid, and a Myc-tagged PGC-1 $\alpha$  plasmid. Both an anti-PGC-1 $\alpha$  and an anti-Myc antibody were used. The results indicate that PGC-1 $\alpha$  was successfully overexpressed (Figures 20 and 21).



**Figure 23:** Western Blot of PGC1- $\alpha$  with anti-Myc antibody. L: ladder; NC: untransfected negative control; PGC-1 $\alpha$   $\approx$  105 kDa

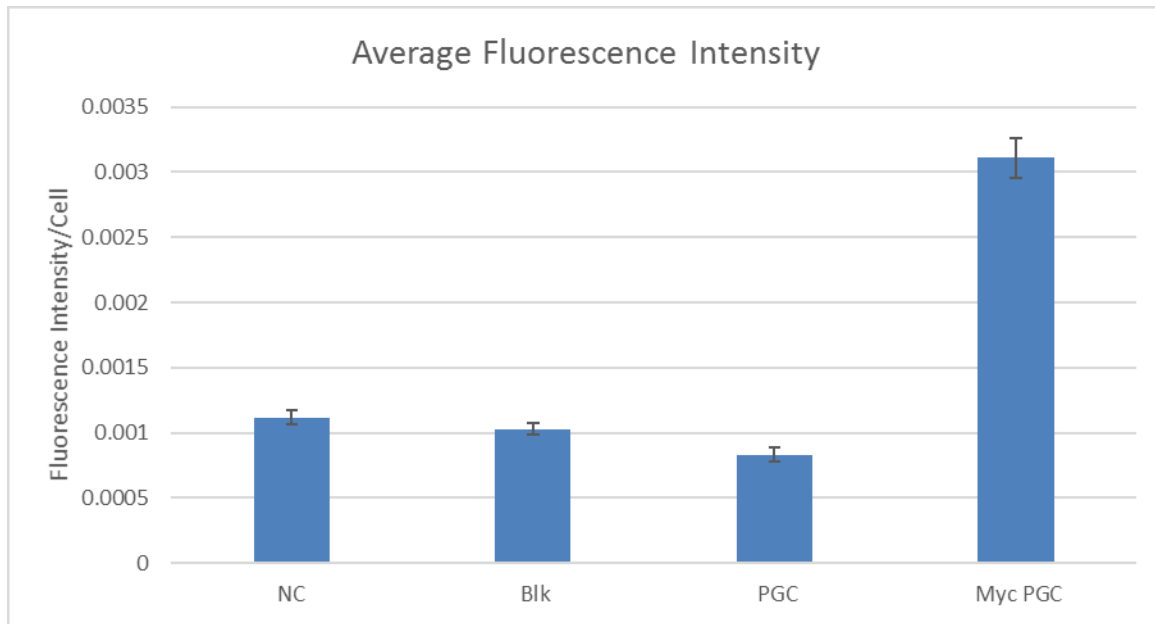


**Figure 24:** Western Blot of PGC1- $\alpha$  with anti-PGC1- $\alpha$  antibody. L: ladder; NC: untransfected negative control; PGC-1 $\alpha$   $\approx$  105 kDa

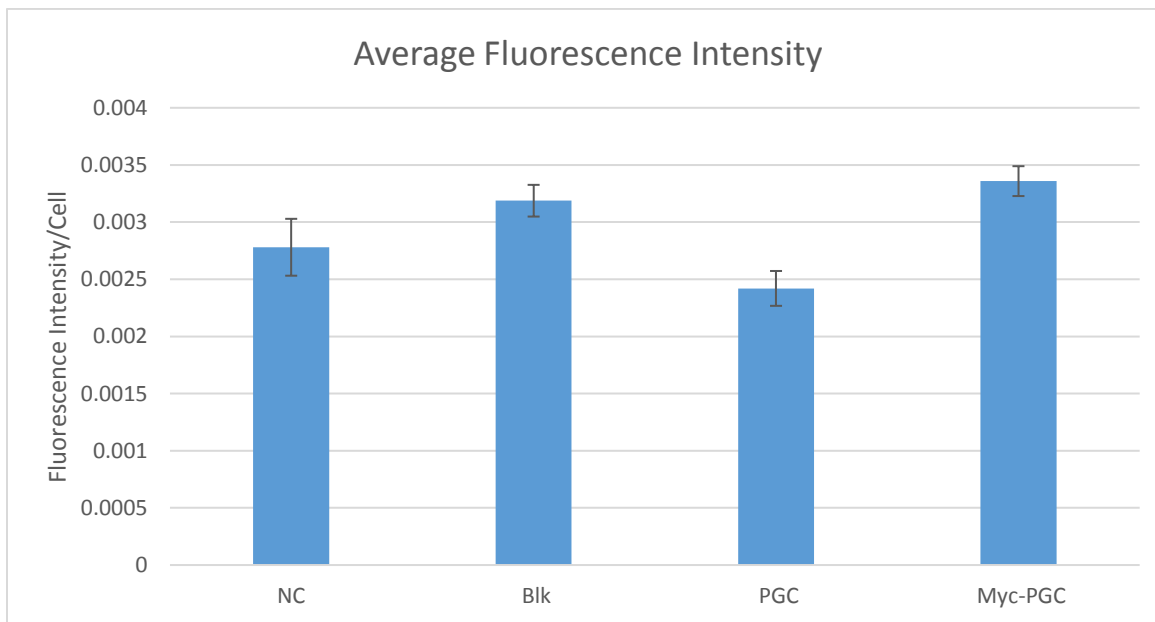
### 4.3.2 Mitotracker

The first attempt at this experiment produced much higher fluorescence values per cell for the cells transfected with the Myc PGC-1 $\alpha$  plasmid when compared to those transfected with a blank plasmid and those not transfected at all (Figure 22). Also, the fluorescence values for the cells transfected with the untagged PGC-1 $\alpha$  plasmid were slightly less than the negative control and blank plasmid transfected HEK cells. This was unexpected as both plasmids had been shown to overexpress PGC-1 $\alpha$  through transfection.

When the mitotracker protocol was repeated, the transfection was done right into the 96 well plate which would be stained in order to ensure no loss of cells during transfer to the new plate which would be read in the plate reader. Once again, the cells transfected with the Myc PGC-1 $\alpha$  plasmid exhibited the highest fluorescence (Figure 23); however, the difference was not very significant when compared to the other conditions. Once again, the untagged PGC-1 $\alpha$  plasmid resulted in the lowest fluorescence intensity.



**Figure 25:** MitoTracker Red Protocol Trial 1 Results



**Figure 26:** MitoTracker Red Protocol Trial 2 Results

## 4.4 Discussion

The Western blots demonstrated that PGC-1 $\alpha$  was successfully overexpressed using both plasmids. The untagged PGC-1 $\alpha$  plasmid resulted in a band consistent with PGC-1 $\alpha$  overexpression with a much stronger band than the untransfected negative control along with the location of the band being consistent with the size of PGC-1 $\alpha$  being approximately 105 kDa. In addition, the Myc tagged PGC-1 $\alpha$  plasmid also resulted in expression as evidenced by the strong band around 105 kDa consistent with the correct size. However, upon creation of a stable pool, PGC-1 $\alpha$  overexpression was not detected through Western blot.

The MitroTracker Red protocol suggests that cells overexpressing PGC-1 $\alpha$  may be creating more mitochondria as evidenced by the higher fluorescence intensity values; however, when the experiment was repeated, the differences between the Myc tagged PGC-1 $\alpha$  transfection and the untransfected negative control and blank plasmid transfection were not as evident. In addition, the cells transfected with the untagged PGC-1 $\alpha$  plasmid consistently exhibited the lowest fluorescence intensities. It should not be concluded that PGC-1 $\alpha$  overexpression makes more mitochondria through a transient transfection, but perhaps it does over time with a stable transfection. There may also be problems with overexpression of PGC-1 $\alpha$  through such a simple vector that results in poor expression.

## Chapter 5: Conclusion

The objective was to work towards engineering a cell line with increased citric acid cycle activity without dependence on essential amino acid from culture medium. To do so, PGC-1 $\alpha$  was overexpressed into HEK cells through a transient transfection and it was determined that there may be an increase in mitochondrial biogenesis. Even though generation of a stable pool from PGC-1 $\alpha$  transfection was achieved, there was no evidence of PGC-1 $\alpha$  overexpression through Western blot.

In addition, a synthetic biology approach was taken in order to begin constructing the biosynthetic pathway for isoleucine and valine through the expression of ILV1, ILV2, ILV3, ILV5, and ILV6. In addition, ILV1 activity was verified through GC-MS analysis and the first metabolite in the isoleucine-valine pathway, oxobutanoate, was produced upon transient transfection in HEK cells. In addition, the presence of amino-acid transaminase in mammalian cells was confirmed through the presence of isoleucine, valine, and leucine precursors (kIle, kVal, and kLeu respectively).

Furthermore, viability studies were performed to further verify the expression of the threonine biosynthetic pathway in a U-2 OS cell line. It was found that normal U-2 OS cells could not withstand the extended absence of threonine. More viability studies were performed in HEK cells to determine sustainability in the absence of four essential amino acids—threonine, isoleucine, leucine, and valine. HEK cells demonstrated a large decrease in viability while lacking these amino acids, but they appeared to not be as adversely affected as U-2 OS cells when lacking essential amino acids. This could prove to be useful if essential amino acid depletion is used as the selection method for construction of the isoleucine, valine, threonine, and leucine pathways into an HEK cell line due to their robustness.



## Chapter 6: Future Work

### 6.1 Completion of Isoleucine/Valine pathway in HEK cells

Further work should focus on advancing the biosynthetic pathway of isoleucine and valine past oxobutanoate and proving metabolite formation. In addition, it may be valuable to repeat the creation of a threonine prototrophic cell line but in HEK<sub>2</sub> cells because they are more industrially relevant for the biopharmaceutical industry and were shown to be more robust when cultured with amino acid limited media.

### 6.2 Metabolic Flux Analysis in U-2 OS Threonine Prototrophic Cell Line

Elucidating the entire threonine biosynthetic pathway through <sup>13</sup>Carbon labeling in the U-2 OS prototrophic cell line would give a novel view of the pathway into a human cell line. It would help guide further efforts to create a cell line capable of producing its own essential amino acids by knowing which amino acids are secreted and consumed the most. Furthermore, other selection methods could be explored in order to find higher threonine producing clones.

### 6.3 Stable Pool of HEK Cells Overexpressing PGC-1 $\alpha$

The next step would be to produce a stable pool of HEK cells overexpressing PGC-1 $\alpha$  and verifying through Western blot. Furthermore, the use of the MitoTracker protocol could show a significant increase in the mitochondrial content of cells overexpressing PGC-1 $\alpha$ . Upon completion of a stable pool that is shown to increase mitochondrial biogenesis, high producing clones can be selected along with performing a metabolic assay to observe the differences in metabolism between normal HEK cells and those overexpressing PGC-1 $\alpha$ .

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